Research article

28S rRNA molecule post processing occurs in two species of planarians (*Girardia tigrina* and *Girardia* sp.) possibly due to a gap deletion

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Citation: Penha, BR; Oliveira, MS; Lopes, KAR; Morais, FV; Campos Velho, NMR. 28S rRNA molecule post processing occurs in two species of planarians (*Girardia tigrina* and *Girardia* sp.) possibly due to a gap deletion. Revista da Biologia, 2022. Vol.22p1-13.
DOI: 10.11606/issn.1984-5154.v22p7-13

Editors: Juliana S. de Barros and Carolina R. Cordula.
Desing: Henrique R. Vieira

Received: 15 November 2019
Accept: 10 March 2021

Abstract: Several studies have shown atypical migration of the 28S rRNA molecule, when exposed to heat denaturation dissociates into two subunits (28Sα and 28Sβ), this event is due to a 'gap deletion' in the molecule. In the majority of the organisms that present the gap region, rRNA degradation has been correlated to a UA-rich conserved segment, located inside a loop of the secondary structure predicted for this region of the 28S rRNA, and upstream of a conserved 5´-CGAAAGGG-3´motif. We showed that this event, the atypical 28S rRNA molecule migration, occurs in planarians *Girardia tigrina* and *Girardia* sp.. The presence of these conserved motifs in the 28S rRNA molecule of *Girardia tigrina* was confirmed. The UA-rich segment was absent. The 5´-CGAAAGGG-3´ motif was present, indicating that the dissociation 28S rRNA is independent of the UA-rich signal. We propose the existence of a putative secondary structure for the D7a segment of the 28S rRNA molecule, and suggest that the atypical RNA profile observed may occur across the *Girardia genus*.

Keywords: Gap region; ribosomal RNA; secondary structure.

1. INTRODUCTION

The most usual method for evaluating the quality of extracted RNA is the analysis of the migration pattern and the intensity of the 18S and 28S ribosomal RNA (rRNAs) bands in eukaryotes, and the 16S and 23S in prokaryotes after agarose or polyacrylamide gel electrophoresis. An intact RNA sample is generally defined by exhibiting clear and distinct bands for both the 18S and the 28S rRNA molecules in eukaryotes (Sambrook et al.,1989).

Before electrophoresis, RNA samples were usually heated and treated with denaturing agents to improve the migration pattern. Denaturing agents are added to gels to better visualize the 18S and 28S rRNAs, which are the most abundant RNA molecules. For this purpose, different denaturants, such as methyl mercury hydroxide, urea, formamide, glyoxal/dimethyl sulfoxide (Rio 2015a), and formaldehyde (Rio, 2015b; Goda and Minton, 1995), can be employed. The major drawback to most of these denaturants is that they require handling restrictions due to their high toxicity and volatility. Therefore, scientists are currently using guanidine thiocyanate (GT), which is less toxic and less volatile, and does not require the use of an extraction hood (Goda and Minton, 1995).

Usually, RNA molecules are kept in their denatured form in 20 mM GT, after heat treatment. Moreover, GT is also capable of protecting RNA against RNAses (Goda and Minton, 1995; Masek et al., 2005), which is a useful benefit for RNA extraction techniques. In the majority of eukaryotes, the 28S and 18S rRNAs have approximately 4,000 and 2,000 nucleotides, respectively; but, in some animals, the size of these molecules can be smaller. In *Giardia lamblia* the size of the 18S and 28S rRNAs are 1,300 and 2,300 nucleotides, respectively (Edlind and

Chakraborty, 1987). Moreover, two species of planarians, *Dugesia subtentaculata* (Riutort et al., 1993, unpublished GenBank: M58343.1, personal communication) and *Schmidtea mediterranea* (Álvarez-Presas et al., 2008), have about 1,300-nucleotide-long 18S rRNA molecules. Usually, rRNA molecules migrate separately under denaturing gel conditions (Skrypina et al., 2003). However, in some groups of insects, researchers detected that under heat denaturing treatment, 28S, and 18S rRNA molecules comigrate in a single band of around 2,000 nucleotides, due to the break and dissociation of the 28S rRNA molecule into two fragments, called 28Sα and 28Sβ (Winnebeck et al., 2010; Sun et al. 2012).

Several studies have shown that the underlying rRNA breaking mechanism is commonly associated with a UA-rich conserved region (5´-UAAU-3´) present in the 28S rRNA molecule. This event, initially described as a "hidden break" (Fujiwara and Ishikawa, 1986; Ogino et al., 1990), occurs approximately 10 bases before the 5' region of the 28Sβ fragment of the 28S rRNA. According to Ware et al. (1985), however, this phenomenon would be better described as "gap deletion", because fragmentation occurs due to the excision of a short sequence from the 28S rRNA precursor, possibly due to a post-processing mechanism. The resulting 28S rRNA molecule is, thus, composed of two fragments, α, and β, that are kept together by non-covalent hydrogen bonds. The dissociation between α and β 28S fragments is due to the heat treatment applied to the RNA sample (Macharia et al., 2015) regardless of whether this is followed by chemical gel denaturation (Winnebeck et al., 2010). McCarthy et al. (2015) showed that the migration profile of the total RNA of many non-insect arthropods in formaldehyde-denaturing agarose gel electrophoresis conditions without previous heat RNA treatment, does not demonstrate the 28S rRNA fragmentation.

When studying the expansion segment D7a of *Aedes albopictus*, Kjer et al. (1994) did not find the proposed 5´-UAAU-3´ processing sequence upstream of the conserved 5´-CGAAAGGG-3´ motif (Fujiwara and Ishikawa, 1986). This finding was also confirmed in other organisms that presented 28S rRNA thermal dissociation but did not harbor the proposed 5´-UAAU-3´ cleavage signal (Sun et al., 2012; Gillespie et al., 2005).

Although the size of the gap region may vary between organisms, this sequence is part of the same D7a expansion segment of metazoan 28S rRNA (**Fig. 1**) (Gillespie et al., 2006; Sun et al., 2012).

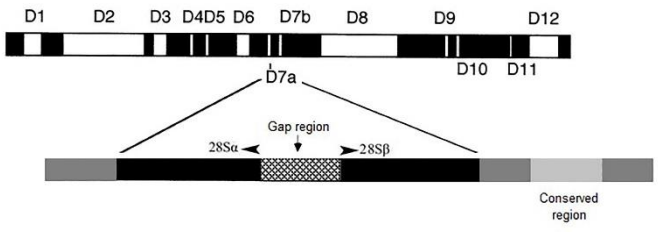

Fig. 1: **Structure of the *28S* rRNA gene.** Top: Black boxes, conserved regions. White boxes, divergent D1–D12 domains. Bottom: scheme of the 176 nucleotides comprising the D7a expansion segment (black boxes) and its flanking regions (gray boxes). The hatched region indicates the site of gap processing inside the D7a expansion segments. The arrows indicate the sites of dissociation of the *28S* rRNA into two fragments, *28S*α and *28S*β. The localization of the conserved 5´CGAAAGGG 3´ motif is indicated (light gray box). Adapted from Mellen et al. (1999).

While gap deletion in 28S rRNA was initially thought to be limited to insects (Ogino et al., 1990; Winnebeck et al., 2010), it has also been identified in a few other organisms such as *Schistosoma mansoni* (van Keulen et al., 1991), *Artemia partogenetica* and *Dugesia japonica* (Sun et al., 2012), and most recently in species representing all clades of non-insect Arthropods, Crustacea, Chelicerata and Myriapoda (McCarthy et al., 2015).

Dugesia japonica is a planarian species of the Dugesiidae Family that also harbors other genera, such as *Girardia*, *Neppia*, *Cura*, *Schmidtea*, and *Recurva* (Solà et al., 2015).

Here we report that the atypical 28S rRNA migration profile also occurs in two species of the planarian *Girardia* genus, *Girardia tigrina* a globalized species, and *Girardia* sp. a species found in Brazil, which is under study as a new species (Oliveira et al., 2018). They were collected in southeastern Brazil's Paraiba Valley region. These results suggest that this phenomenon may occur across the *Girardia* genus and possibly the whole Dugesiidae Family, given its occurrence in *Dugesia japonica*.

2. MATERIAL AND METHODS

2.1 Planarian specimens

All specimens of limnic planarians were collected in southeastern Brazil's Paraiba Valley region. *Girardia tigrina* and *Girardia* sp. were grown at the Planarian Laboratory (LAPLA) of the University of Paraiba Valley, São José dos Campos, Brazil. Before RNA extraction, randomly selected specimens were starved for 15 days (Garcia-Fernandéz et al., 1991; Oliveira et al., 2018) to avoid contaminant or mucus production.

2.2 RNA Extraction

RNA of two specimens from both planarian species were extracted using the TRIzol® method (Invitrogen™). The cells were mechanically lysed by vigorous shaking for 10 min with one volume of glass beads in the presence of 2.5 volumes of TRIzol® reagent, followed by 10 min incubation at room temperature and centrifugation (12,000xg, 5 min, 4°C) to remove cell debris. For each 1 ml of the recovered supernatants, 0.2 ml of chloroform (Synth) was added, followed twice by phenol:chloroform:isoamyl alcohol extractions (Sigma) and a final chloroform extraction. A 0.5 volume of salt solution (sodium citrate 1.2 N and sodium chloride 0.8 N) and 0.5 volume of isopropanol (Synth) were added to the supernatants. The RNA precipitate was collected after an overnight incubation at -20°C, by centrifugation (12,000xg, 20 min, 4°C), washed once in 1 ml 80% ethanol (Synth), dried, suspended in 50 µl of sterile water, and stored at -80°C.

2.3 Total RNA Yield and Purity

The yield and quality of the total RNA extracted were determined using a NanoDrop ND 1000 Spectrophotometer (Thermo Scientific). The ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) was used to assess the purity of the sample.

2.4 RNA sample preparation

One microgram of the extracted total RNA from the two planarian specimens *Girardia tigrina* and *Girardia* sp. was submitted to different heat treatments to evaluate the *28S* rRNA molecule dissociation in denaturing and non-denaturing gel electrophoresis conditions. RNA samples were either 1) not heated, 2) heated for 1 minute at 70ºC or 3) heated for 10 minutes at 70ºC. After the incubation time, the RNA samples were kept on ice, and applied to a non-denaturing or formamide-denaturing loading buffer (Invitrogen™), and were applied to agarose gel.

2.5 Denaturing and Non-denaturing Agarose Gel Electrophoresis

RNA samples were visualized in 1.0% agarose gels in 1X TBE buffer (Tris-borate-EDTA) (Sigma) impregnated with 0.5 $\mu g.ml^{-1}$ ethidium bromide (Sigma), either with 20 mM guanidine thiocyanate (Sigma, denaturing gel) or without GT (non-denaturing gel).

RNA migration was photo-documented after gel electrophoresis (80V for 1 hour) under UV light (Bio-Imaging Systems Visible and Ultraviolet).

2.6 Multiple Sequence Alignment

The 176-nucleotide fragment of the *28S* rDNA fragment from *Girardia tigrina* (GenBank: U78718.1) and *Dugesia japonica* (GenBank: JF827607.1), used in Sun et al. (2012), and analyzed in this work is situated at the region between nucleotides 1708 and 1883 based on the *Girardia tigrina 28S* rDNA sequence, and comprehends the region of the D7a expansion sequence, formed by 76 nucleotides and is located between nucleotides 1722 and 1799 based on the *Girardia tigrina 28S* rDNA sequence. Sequences of both *Girardia tigrina* and *Dugesia japonica* were obtained from the GenBank database.

Multiple sequence alignment was performed using the 7.2.5 version of the BioEdit Sequence Alignment Editor (Hall, 1999).

2.7 Secondary Structure Determination

The secondary structure of the D7a expansion sequence of *28S* rRNA was predicted using the Fold algorithm of the RNAstructure web server (Mathews et al., 2004; Reuter and Mathews, 2010; Bellaousov et al., 2013), which is an improved algorithm based on free energy minimization and dynamic programming.

All molecular experiments were done at the Laboratory of Cellular and Molecular Biology of Fungi of the University of Paraiba Valley, São José dos Campos, Brazil.

3. RESULTS

Girardia spp. ***28S rRNA migration profile***

Total RNA from two specimens of both planarian species *Girardia tigrina* and *Girardia* sp., was measured after purification, and only samples with absorbance at 260 to 280 nm ratio values ($A_{260/280}$) above 2.0 (Seetin and Mathews, 2012) and concentrations ranging between 300-400 $ng.ml^{-1}$ were used in further experimentation.

The planarian *18S* and *28S rRNA* molecule profiles were visualized in agarose gel electrophoresis with one microgram (1 µg) from each RNA sample. The RNA samples of *G. tigrina* and *Girardia* sp. that were heated to 70°C for 1 minute or 10 minutes, and loaded on agarose gels with a non-denaturing loading buffer showed a single band in both gel conditions, under non-denaturing (without GT) after the 10-min heat treatment (**Fig. 2A**), and denaturing (GT) after 1 min heat treatment (**Fig. 2C**).

In contrast, non-heat-treated RNA samples presented the *18S* and *28S* rRNA individual bands, both under denaturing and non-denaturing agarose gel conditions, with the band corresponding to *18S* rRNA being more intense when compared to the *28S* rRNA band (**Fig. 2B**).

Sequence analysis of the D7a segment of 28S rDNA molecule from *Girardia tigrina*

To evaluate if *Girardia tigrina* presented the same *28S* rDNA gap region as that described in *28S* rRNA dissociation of *Dugesia japonica* and other eukaryotes, we aligned and compared the 176 nucleotides flanking the D7a expansion segment from *28S* rDNA of both *Dugesia japonica* and *Girardia tigrina* (**Fig. 3**). Sequences of both *Girardia tigrina* and *Dugesia japonica* were obtained from the GenBank database (see material and methods).

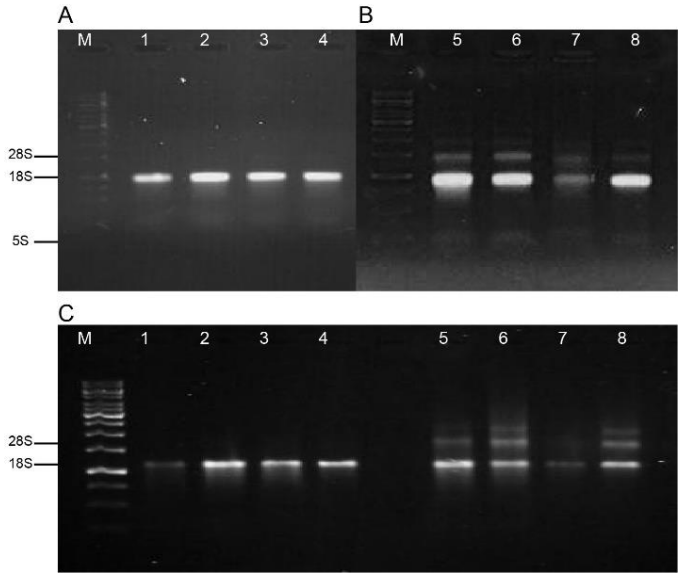

Fig. 2: **Migration profile of total RNA extracted from *Girardia tigrina* and *Girardia* sp**. RNA samples were submitted to electrophoresis on 1% agarose gel impregnated with ethidium bromide. A, heat treated for 10 min (lanes 1, 2, 3 and 4) and B, non-heat treated (lanes 5, 6, 7 and 8) RNA samples under non-denaturing gel conditions. C, heat treated for 1 min (lanes 1, 2, 3 and 4) and non-heat treated (lanes 5, 6, 7 and 8) RNA samples under denaturing gel conditions, containing 20 mM guanidine thiocyanate. Lanes 1, 2, 5 and 6: *Girardia sp.* Lanes 3, 4, 7 and 8: *Girardia tigrina*. M: GeneRuler 1 kb ladder (Thermo Scientific™). The *28S*, *18S* and *5S* rRNA migration bands are identified.

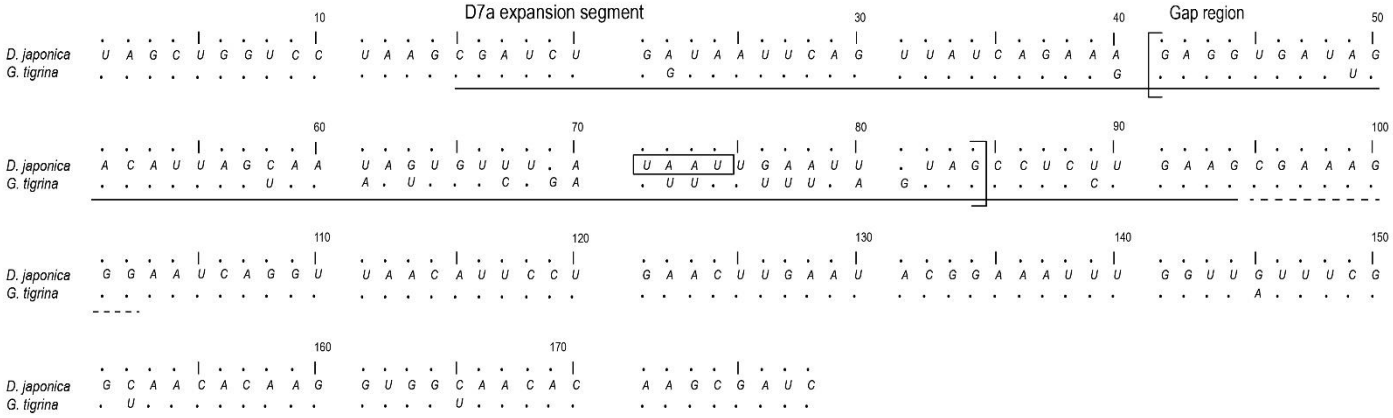

Figure 3: //**Alignment of the *28S* rDNA D7a expansion segment and flanking regions of *Dugesia japonica* and *Girardia tigrina*.** The aligned segment containing 176 nucleotides along with the D7a expansion segment is underlined; the conserved 5´-CGAAAGGG-3´ motif is shown by a dashed line; and the gap region is between brackets. The 5´-UAAU-3´ motif (box inside the gap region) is absent in *Girardia tigrina*. Both *28S* rDNA sequences were obtained from the GenBank database.

After alignment, the sequences showed 88% identity. Moreover, the AU-content of the *Girardia tigrina* D7a expansion segment was 65.8% and, thus, very close to the 67.9% AU-content already described by Sun et al. (2012), for *D. japonica*. The AU-content of the gap region of *Girardia tigrina* and *D. japonica*, were 74.4% and 69.8%, respectively. The conserved motif 5´-CGAAAGG -3´ proximal to the 3′ end of the gap region found in *Dugesia japonica*, as well as in some eukaryotes (Sun et al., 2012), was also conserved in *Girardia tigrina* (**Fig. 3**, dashed line), but the UAAU-rich segment was absent (**Fig. 3**, box). In the figure, the flanking nucleotides outside the brackets represent the dissociation site of the *28S* molecule in α and β subunits in *D. japonica*, suggesting the putative dissociation site in *Girardia tigrina*.

Predicted secondary structure of the D7a segment of the 28S rRNA molecule from Girardia tigrina

Several studies suggest that a secondary structure of the RNA may play a role in *28S* rRNA gap deletion. Therefore, two possible secondary structures for the D7a expansion segments of *Girardia tigrina* were predicted using the fold free energy minimization algorithm (**Fig. 4 A-B**) of the RNAstructure package (Bellaousov et al., 2013). Common to these two structures is the presence of hairpin loops (**Fig. 4A-B**). This hairpin loop has, however, alternative conformations. Shortening this sequence to the Gap region conserves both alternative conformations of this loop. The suggested structures seem, furthermore, to be conserved in the *Dugesia japonica* sequence, as shown in Figure 4C.

According to the employed method, both secondary structures have close values of calculated free energy, but among the two possibilities (**Fig. 4A-B**), the most probable secondary structure for the *Girardia tigrina 28S* rRNA D7a expansion segment is shown in **Fig. 4B**, having the lowest free energy (-22.7 kcal.mol^{-1}). The other predicted structure shown in **Fig. 4A**, has a calculated free energy of -22.3 kcal.mol^{-1}. The boxed region of the secondary structure shows the conformation of the gap region (**Fig. 4B**).

Furthermore, in comparison with *D. japonica* (**Fig. 4C**, free energy -22.8 kcal.mol^{-1}), the putative secondary structure for the D7a segment for *Girardia tigrina* shown in Figure 4B, is visually very similar.

DISCUSSION

Girardia spp. *28S rRNA migration profile*

The unusual single band comigration profile of 18S and 28S rRNA reported in several eukaryotes (Ogino et al., 1990; van Keulen et al., 1991; Winnebeck et al., 2010; Sun et al., 2012; McCarthy et al., 2015), also occurs in two species of the *Girardia* genus, *G. tigrina* and *Girardia sp*., after thermal induction, in agreement with results demonstrated in earlier studies from other species (Ogino et al., 1990; van Keulen et al., 1991; Winnebeck et al., 2010; Sun et al., 2012; McCarthy et al., 2015).

All heat-treated RNA samples exhibited a unique band migration profile under both agarose gel electrophoresis conditions. On the other hand, all RNA samples that were not heat denatured exhibited the usual RNA migration profile, with *18S* and *28S* rRNA molecules migrating as individual bands both under non-denaturing and denaturing agarose gel electrophoresis conditions, as expected in the majority of organisms (Sambrook et al., 1989). Notwithstanding, in non-heated RNA samples, the *18S* rRNA band was more intense if compared to the *28S* rRNA band, differing from a typical rRNA profile. These observations regarding the RNA migration profile in *Girardia tigrina* and *Girardia sp.* corroborate similar observations in another planarian species, *Dugesia japonica* (Sun et al., 2012), as well as in insects (Macharia et al., 2015) and non-insect arthropods (McCarthy et al., 2015).

This migration phenomena happens due to the deletion of a gap (Ware et al. 1985), also referred as “hidden break” (Fujiwara and Ishikawa, 1986) in the *28S* rRNA sequence, that results in two fragments of the *28S* rRNA molecule when exposed to heat.

According to Winnebeck et al. (2010), in *Apis mellifera* and most insects the *28S* rRNA consists of two

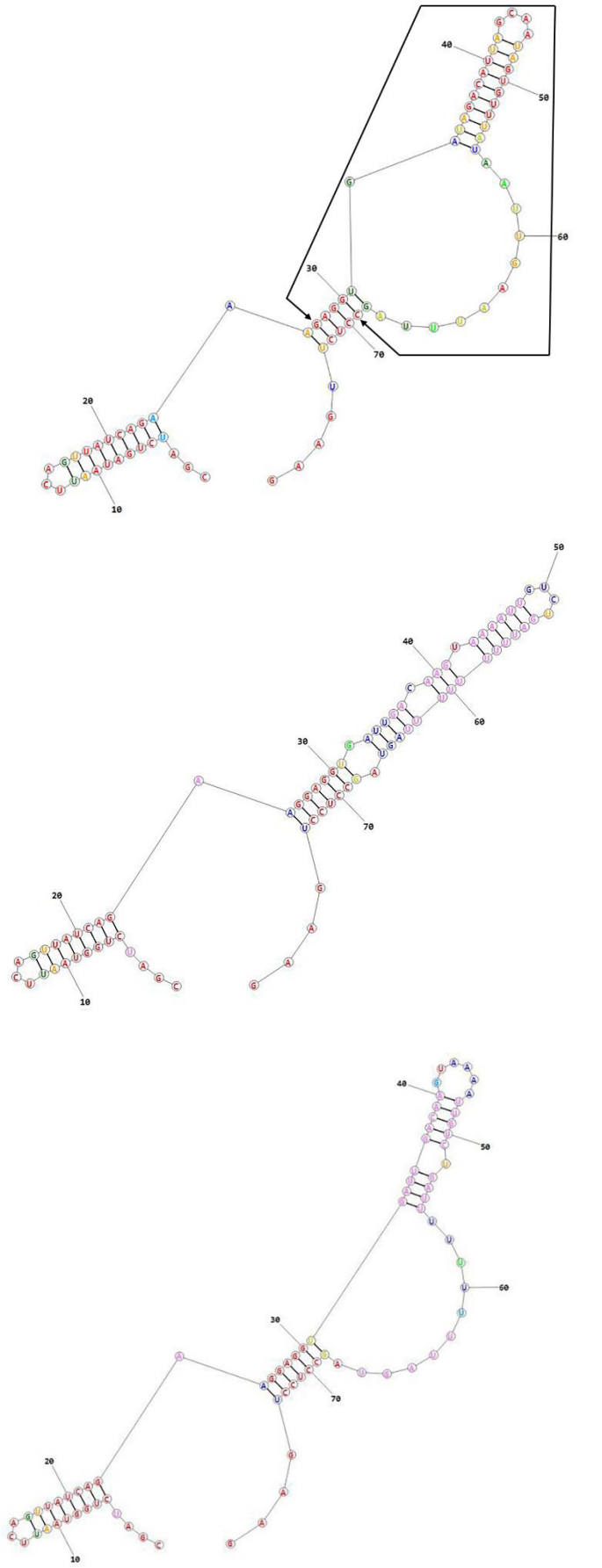

Fig. 4: **Putative secondary structure models for the D7a expansion segment of the *28S* rRNA of *Girardia tigrina* and *Dugesia japonica*.** The free binding energy calculated for each structure is shown above each putative secondary structure: A, -22.3 kcal.mol^{-1}; B, -22.7 kcal.mol^{-1}; C, -22.8 kcal.mol^{-1}. A and B refer to the two putative structures of the 28S rRNA's D7a expansion segment from G. *tigrina* and C refer to the same structures in D. *japonica*. In B, the putative post processing sites are indicated by arrows; the 3′ end of the *28Sα* fragment and the 5′ end of the *28Sβ* fragment are shown using the symbols *α*3′

separate fragments that are hydrogen-bonded together. Heat treatment at 70 °C for 2 minutes disrupts these hydrogen bonds and the *28S* rRNA fragment dissociates, generating two fragments (*α* and *β*) that are similar in size to the *18S* rRNA molecule.

The results obtained in this study are very similar to those observed for *Apis mellifera* (Winnebeck et al., 2010) despite the different procedures used. The total RNA migration profile of *Girardia tigrina* and *Girardia sp*. was analyzed by traditional denaturing and non-denaturing agarose gel electrophoresis in our study while in *Apis mellifera*, the RNA migration profile was analyzed by using an Agilent 2100 Bioanalyzer that consists of a microfluidic capillary electrophoresis system that is able to define the size of the fragments more accurately.

Comigration of *18S* rRNA and *28S* rRNA fragments *α* and *β,* in *Girardia tigrina* and *Girardia sp.* occurred after heat denaturation at 70 °C for 1 minute on a denaturing gel. This resulting migration profile was not detected by gel electrophoresis in non-heated RNA samples even under guanidine thiocyanate denaturing condition.

Comigration of *18S* rRNA and *28S* rRNA fragments *α* and *β,* in *Girardia tigrina* and *Girardia sp.* occurred after heat denaturation at 70 °C for 1 minute on a denaturing gel. This resulting migration profile was not detected by gel electrophoresis in non-heated RNA samples even under guanidine thiocyanate denaturing condition.

Comigration of *18S* and *28S* rRNA molecules after heat denaturing conditions was also observed in *Bombyx mori* (Fujiwara and Ishikawa 1986), *Schistosoma mansoni* (van Keulen et al., 1991), *Trichinella spiralis* (Zarlenga and Dame, 1992), *Artemia parthenogenetica* (Sun et al., 2012), *Dosima fascicularis*, *Lepas anatifera*, *Pollicipes pollicipes*, *Scolopendra subspinipes* (McCarthy et al., 2015), with several South American rodent species of the genus Ctenomys (Melen et al., 1999) also exhibiting this, the only Deuterostome example of this phenomenon.

Sequence analysis of the D7a segment of the 28S rDNA molecule from Girardia tigrina

In this study we showed a high correlation (88% of 176 nucleotides) between the sequence corresponding to the D7a expansion segment of the *28S* rDNA of *Dugesia japonica* (Sun et al., 2012) and *Girardia tigrina*, with an AU content of 67.1% and 63.3%, respectively. The putative gap region of *Girardia tigrina* displayed a high UA level, but did not have the 5´-UAAU-3´ sequence present in the *Dugesia japonica* gap region (Sun et al., 2012). According to Fujiwara and Ishikawa (1986) and Ogino et al. (1990) the presence of the 5´-UAAU-3´ sequence followed by the 5´CGAAAGGG 3´ conserved sequence in the D7a segment characterized the gap processing event in organisms. However, the occurrence of *28S* rRNA gap processing in organisms with no 5´-UAAU-3´ sequence such as *Artemia parthenogenetica* (Sun et al., 2012) and *Aedes albopictus* (Park and Fallon, 1990) corroborate the results for *Girardia tigrina* and *Girardia* sp. *Artemia parthenogenetica* and *Aedes albopictus* presented a *28S* rRNA AU content of 46.5% and 65.3%, respectively. The latter is similar to the AU

content of *Girardia tigrina* (63.3%). All species contained the 5´CGAAAGGG 3´ conserved sequence.

In the *28S* rRNA secondary structure of *Dugesia japonica* (Sun et al., 2012) as well as in *Sciara coprophila* (Ware et al., 1985) and *Schistosoma mansoni* (van Keulen et al., 1991) the flanking regions of the D7a expansion segment, which is extremely AU-rich, may form a stem loop structure, containing the putative 5´-UAAU-3´ cleavage site within the loop. However, the lack of the 5´-UAAU-3´ motif did not affect the fragmentation of the *28S* rRNA molecule in *Artemia parthenogenetica* (Sun et al., 2012) and in *Girardia tigrina*, as shown in this study.

Sun et al. (2012) suggested that gap processing probably is associated with the recognition of the stem loop secondary structures instead of the presence of conserved motifs, since the dissociation of the two *28S* rRNA fragments were not affected in organisms lacking those motifs, thereby suggesting that the secondary structure of 28S rRNA molecules are formed prior to the cleavage of the 28S rRNA.

Predicted secondary structure of the D7a segment of the 28S rDNA molecule from Girardia tigrina

There are several methods that can be used to predict the secondary structure of RNA molecules. The most commonly used method is based on free energy minimization and consists in selecting the sets of pairs of complementary bases resulting that form the energetically most stable molecules (Seetin and Mathews, 2012). Two secondary structure possibilities were generated for the D7a segment of *Girardia tigrina 28S* rDNA. The most probable secondary structure showed a free energy of -22.7 kcal.mol-1.

CONCLUSION

We suggest that, under heat treatment, *28S* rRNA molecules from *Girardia tigrina* and *Girardia* sp. dissociate into two subunits (α and β) that comigrate with the *18S* rRNA molecules, exhibiting a single band that is typically observed in the rRNA profile of insects and some other organisms. Moreover, this event is independent of denaturing agarose gel electrophoresis, reinforcing the thermolability of the *28S* rRNA. This study opens up new questions for further analysis of the phenomenon known as gap deletion.

The molecular mechanism that promotes the dissociation of the *28S* rRNA molecules in planarians, as well as in other organisms is still unknown and more studies are necessary to unravel it both molecularly and functionally.

Inasmuch as *28S* rRNA molecules from both *Girardia tigrina* and *Girardia* sp. dissociate into two subunits, this event may occur across the genus *Girardia* and, given that it is also present in *D. japonica*, may even occur across all the Dugesiidae Family.

Competing interests
No competing interests declared

Funding
This research received no specific financial aid from any funding agency of the public, commercial or not-for-profit sectors.

Data availability
Accession numbers for *Girardia tigrina* and *Dugesia japonica* 28S rDNA are U78718.1 and JF827607.1 respectively.

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